REMARKS

Claims 1-8 are pending. Claims 1, 3, 5, and 7 have been examined and stand rejected. Claims 1-6 and 8 have been canceled. Claim 7 has been amended. Claims 9-20 have been added. No new matter has been introduced. Reconsideration and allowance of Claims 7 and 9-20 is respectfully requested.

The Rejection of Claims 1, 3, 5, and 7 as Being Unpatentable over Claims 1-4 of U.S. Patent No. 6,743,172

Applicants will file a terminal disclaimer in compliance with 37 C.F.R. 3.73(b) upon the allowance of claimed subject matter in the instant application.

The Rejection of Claims 1, 3, 5, and 7 Under 35 U.S.C. § 112, Second Paragraph (Indefiniteness)

Claims 1, 3, 5, and 7 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite in their recitation of "enhancing." The Examiner has taken the view that the term "enhancing" is a relative term which renders the claim indefinite.

Claims 1, 3, and 5 have been canceled. Without acquiescing to the Examiner's position, but in order to facilitate prosecution, Claim 7 has been amended to replace the term "enhancing" with the term "increasing." Support for this amendment is found throughout the application as filed, for example, at page 6, lines 20-21 and lines 26-30; page 8, lines 15-17; page 19, line 28, to page 20, line 6; and page 54, line 16, to page 56, line 12. Removal of this ground of rejection is respectfully requested.

The Rejection of Claims 1, 3, 5, and 7 Under 35 U.S.C. § 112, First Paragraph (Enablement)

Claims 1, 3, 5, and 7 stand rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement.

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As an initial matter, it is noted that Claims 1, 3, and 5 have been canceled. Without acquiescing to the Examiner's position, but in order to facilitate prosecution, Claim 7 has been amended and now recites:

> 7. (Currently amended) A composition for eliciting or increasing the titer of antibodies specific for a cell surface receptor antigen, comprising:
>
> a) a first recombinant expression construct containing at least one promoter operably linked to a nucleic acid sequence encoding a cell surface receptor antigen comprising a transmembrane domain and a cell surface receptor domain that binds to at least one of a cytokine, a chemokine or a growth factor;
>
> b) a second recombinant expression construct containing at least one promoter operably linked to a nucleic acid sequence encoding a first immune response altering molecule; and
>
> c) a nucleic acid sequence encoding a second immune response altering molecule;
>
> wherein said first immune response molecule is 4-1BB-ligand and said second immune response altering molecule is selected from the group consisting of CD80/B7.1 and CD86/B7.2.

Support for Claim 7, as amended, is found throughout the specification as filed, for example at page 7, line 25, to page 10, line 19; page 23, line 17, to page 24, line 6; and page 50, line 24, to page 58, line 12.

The Examiner acknowledges that that specification is enabling for a vaccine composition comprising one or more expression constructs encoding Her2/neu, CD86/B7.2 and 4-1BB-ligand. However, the Examiner asserts that the specification does not reasonably provide enablement for other vaccine compositions for eliciting or enhancing the titer of antibodies for any cell surface receptor antigen, wherein the vaccine composition comprises one or more recombinant expression constructs encoding any cell surface receptor antigen plus any two immune response altering molecules. Applicants disagree with the Examiner's conclusion for the following reasons.

It is submitted that the Examiner has not met the required burden of establishing a reasonable basis to question the enablement provided in the specification for the claimed

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invention, and therefore has not established a *prima facie* case of non-enablement for the claimed invention, as amended.

As stated in the M.P.E.P.:

> A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C.§ 112, first paragraph, unless there is a reasons to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

M.P.E.P. 2164.04 citing *In re Marzocchi*, 439 F.2d 220, 223, 169 U.S.P.Q. 367, 369 (C.C.P.A. 1971) (emphasis in the original).

As noted above, the claimed invention, as amended, is directed to a composition for eliciting or increasing the titer of antibodies specific for a cell surface receptor antigen. The composition comprises a combination of a first recombinant expression construct comprising at least one promoter operably linked to a nucleic acid sequence encoding a cell surface receptor antigen comprising a transmembrane domain and a cell surface receptor domain that binds to at least one of a cytokine, a chemokine or a growth factor; and a second recombinant expression construct comprising at least one promoter operably linked to a nucleic acid sequence encoding 4-1BB-ligand and a nucleic acid sequence encoding a second immune response altering molecule selected from the group consisting of CD80/B7.1 and CD86/B7.2.

It is submitted that the specification enables the full scope of the claims, as amended. As acknowledged by the Examiner, the specification is enabling for a composition comprising one or more expression constructs encoding Her2/neu, CD86/B7.2 and 4-1BB-ligand. It is further noted that the specification is enabling for a composition comprising an expression construct encoding a cell surface receptor antigen comprising a transmembrane domain and a cell surface receptor domain that binds to at least one of a cytokine, a chemokine or a growth factor, and a

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second expression construct comprising 4-1BB-ligand in combination with a nucleic acid encoding either CD80/B7.1 or CD86/B7.2.

For example, as demonstrated in the specification, a composition comprising pLNCX-Rat-Neu+ pLNCX-B7.1 + pLNCX-4-1BBlig (Group 6) and a composition comprising pLNCX-Rat-Neu+ pLNCX-B7.2 + pLNCX-4-1BBlig (Group 7) were injected into mice transgenic for the rat Neu2 transgene. See Example 2, specification at page 56 line 1, to page 54, line 15. The immune sera of the mice obtained before and after vaccination were analyzed for the presence of antibodies that bound to the surface receptor antigen (in this example the rat Neu protein). As shown in FIGURE 1, and described in Example 3, the sera from the animals of group 6 and group 7 contained significantly elevated levels of anti-Neu antibodies in comparison to the background levels of anti-Neu antibodies observed in sera obtained from the animals prior to vaccination. See FIGURE 1 and specification at page 54, line 16, to page 56, line 12. Therefore, it is demonstrated in the specification as filed that the combination of a first vector encoding a cell surface receptor antigen (rat Neu), and a separate vector encoding the 4-1BB-ligand in combination with either B7.1 or B7.2 is effective for increasing the titer of antibodies for the cell surface receptor antigen.

With regard to the Examiner's assertions that applicants' own examples demonstrate the unpredictability in the art of DNA vaccines, it is noted that the Examiner has relied on the observation that Group 4 mice, (immunized with pLNCX-Rat-Neu + pLNCX-B7.2) exhibited an increase in tumor size, which is not relevant to the claimed invention. Rather, contrary to the Examiner's assertion, the claimed invention is directed to a composition comprising the *combination* of a first vector encoding a cell surface receptor antigen, *a second vector encoding the 4-1BB-ligand and* at least one of B7.1 or B7.2. This claimed combination is demonstrated to generate increased antibody titer to the cell surface receptor antigen, as described above.

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As further evidence that the claimed invention is useful for its intended purpose and provides predictable results, submitted herewith is a journal article describing results obtained by the present inventors using the methods disclosed in the specification as filed. See Disis, M.L., et al., "Plasmid-based Vaccines Encoding Rat neu and Immune Stimulatory Molecules Can Elicit Rat neu-specific Immunity," *Molecular Cancer Therapeutics* 2:995-1002 (2003), attached hereto as Attachment 1. As described in Attachment 1, a plasmid containing pLNCX-rat neu and a single plasmid containing two co-stimulatory molecules (pMG-CD137L/IRES/CD80 or pMG-CD137L/IRES/CD86) (FIGURE 1 D and E) were used to vaccine mice. The CD137ligand corresponds to the 4-1BB-ligand (see the specification at page 13, lines 21-23). As shown in FIGURE 3 of Attachment 1, antibodies to rat neu could not be detected in animals immunized with vector alone, and a significant antibody response was detected in mice vaccinated with pLNCX-rat neu + either CD137L/CD80 or CD137L/CD86. See FIGURE 3, page 998. The study described in Attachment 1 concluded "[p]lasmid-based vaccines encoding neu and CD137L and either CD80 or 86 were effective in generating both an antibody and a T-cell response specific for neu; therefore, tolerance was circumvented." Page 1000, 2nd Col.

The Examiner has taken the view that Eck et al., teaches that the art of gene therapy is unpredictable due to the fate of the DNA vector itself, the trafficking of the genetic material within cellular organelles, and the level of mRNA produced. However, contrary to the Examiner's assertions in this regard, as further described in Attachment 1, after the plasmids were administered to the mice, (either i.m. or i.d.), transcript could be detected in the lymph nodes of the immunized mice even at the lowest dose of plasmid. See Attachment 1, page 997, 2nd Col. Moreover, as further described in Attachment 1, in order to determine whether trafficking cells expressed rat neu protein, immunohistochemical staining of lymph nodes

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derived from mice immunized i.d. with either 30ug of vector alone, or vector containing rat neu was carried out. As shown in FIGURE 2C of Attachment 1, mice receiving plasmid that encoded rat neu had significant expression of the rat neu protein in the cytoplasm of cells in the lymph node at 48h after injection. See Attachment 1, page 998.

With regard to the Examiner's assertion that the specification does not reasonably provide enablement for any cell surface receptor antigen, it is noted that Claim 7 has been amended to clarify that the surface antigen comprises a transmembrane domain and a cell surface receptor domain that binds to at least one of a cytokine, a chemokine or a growth factor. Adequate guidance is provided in the specification to make and use a composition comprising a recombinant expression construct containing at least one promoter operably linked to a nucleic acid sequence encoding a cell surface receptor antigen comprising a transmembrane domain and a cell surface receptor domain that binds to at least one of a cytokine, a chemokine or a growth factor. Numerous examples of cell surface receptor antigens (SRA) are provided in the specification that comprise a transmembrane domain and a cell surface domain that bind to a cytokine, chemokine, or growth factor receptor (see, *e.g.*, page 8, line 23, to page 10, line 3). The specification also provides guidance on the generation of recombinant expression constructs containing nucleic acids encoding surface receptor antigens (see page 32, line 6, to page 44, line 14), including the selection of promoters, (see page 35, line 20, to page 37, line 17), and the use of internal ribosome binding sites (IRES) (see page 37, line 18, to page 38, line 2).

As stated in M.P.E.P. Section 2164.02:

For a claimed genus, representative examples together with a statement applicable to the genus as a whole will ordinarily be sufficient if one skilled in the art (in view of level of skill, state of the art and the information in the specification) would expect the claimed genus could be used in that manner without undue experimentation. Proof of enablement will be required for other members of the claimed genus only where adequate reasons are advanced by the examiner to establish that a person skilled in the art could not use the genus as a whole without undue experimentation

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As described above, the specification provides a working example that demonstrates the successful use of the claimed composition with the representative surface antigen receptor rat neu, which is a homologue of human Her-2/neu. Her-2 is a member of the epidermal growth factor receptor family and is a transmembrane protein which consists of a cysteine rich extracellular domain (the cell surface receptor domain which functions in ligand binding) and an intracellular cytoplasmic domain (which has kinase activity involved in growth regulation), as described in Disis, M.L., et al., *Molecular Cancer Therapeutics* 2:995-1002 (2003) (Attachment 1). Therefore, the results demonstrated with the compositions in the instant application and Attachment 1 using the neu antigen would be expected to be representative of other cell surface antigens comprising a transmembrane domain and a cell surface receptor domain that binds to at least one of a cytokine, a chemokine or a growth factor, as claimed.

It is also noted, contrary to the Examiner's assertion, that adequate guidance is provided in the specification with regard to methods of administration of the composition. For example, detailed guidance is provided for suitable pharmaceutical compositions and dosages for administration to a subject (see specification at page 45, line 20, to page 46, line 16). Detailed guidance is also provided for various routes of administration (see specification at page 46, line 20, to page 48, line 19). Moreover, a working example is provided demonstrating effective delivery of the compositions by intradermal injection into mice (see page 53, line 1, to page 56, line 12).

Therefore, based on the guidance provided in the specification, in view of the knowledge in the art, one skilled in the art would be able to make and use the claimed compositions, which is sufficient to satisfy the enablement requirement. M.P.E.P. Section 2164.01(c). In view of the guidance provided in the specification, the high level of skill in the art and the working examples, any experimentation that would be required to make and use the claimed compositions

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would be merely routine, and not undue. Accordingly, removal of this ground of rejection is respectfully requested.

The Rejection of Claims 1, 3, 5 and 7 Under 35 U.S.C. § 102(e) as Being Anticipated by U.S. Patent No. 6,348,450 (Tang et al.)

Claims 1, 3, 5, and 7 stand rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent No. 6,348,450 (Tang et al.). The Examiner has taken the view that Tang et al. discloses a vaccine composition comprising a single vector encoding human carcinoembryonic antigen (CEA), a co-stimulatory molecule, such as B7-1 or B7-2, and a cytokine, such as GM-CSF. Applicants traverse this rejection for the following reasons.

Claim 7 has been amended as described above. Claims 1, 3, and 5 have been canceled. It is noted that Tang et al. does not disclose the 4-1BB-ligand, and therefore does not anticipate the claimed invention, as amended, for at least this reason. Moreover, Tang et al. does not disclose or suggest a composition comprising a first vector encoding a cell surface receptor and a second vector encoding 4-1BB-ligand in combination with B7-1 or B7-2, as claimed.

Tang et al. is generally directed to compositions and methods for non-invasive immunization. As described in Tang et al., "[t]he genes can be delivered by various methods including device-free topical application or coating the genes on the surface of the skin of an animal by a device such as a pad or bandage, e.g., an adhesive bandage." Col. 17, lines 60-63. In fact, as would be understood by one of skill in the art, the non-invasive topical vaccination methods described in Tang et al. would result in very low efficiency of transduction, which would lead one away from the use of a composition comprising at least two recombinant expression vectors, as claimed.

Therefore, it is demonstrated that Tang et al. does not anticipate or render obvious the claimed invention, as amended. Removal of this ground of rejection is respectfully requested.

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The Rejection of Claims 1, 3, 5 and 7 Under 35 U.S.C. § 103(a) as Being Unpatentable Over Conry et al., *Gene Therapy 3*:67-74 (1996).

Claims 1, 3, 5, and 7 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Conry et al., *Gene Therapy 3*:67-74 (1996). The Examiner characterizes Conry et al. as disclosing co-delivery of genes encoding B7-1 and human carcinoembryonic antigen (CEA) within separate plasmids or a dual plasmid with two independent expression cassettes. The Examiner then asserts that the skilled artisan would have been motivated to deliver a dual plasmid encoding both B7-1 and CEA in combination with a plasmid encoding GM-CSF by gene gun delivery to the skin to achieve an improved antibody response as compared to the delivery of a plasmid encoding DEA alone. Therefore, the Examiner concludes a composition comprising the dual plasmid encoding both B7-1 and CEA and the plasmid encoding GM-CSF would have been obvious. Applicants disagree with the Examiner's conclusions for the following reasons.

It is submitted that the Examiner has failed to establish a *prima facie* case of obviousness because the cited reference fails to teach or suggest all the elements of the invention.

As an initial matter, it is noted that Claim 7 has been amended as described above. Conry et al. does not teach or suggest the use of 4-1BB-ligand, as claimed. Therefore, Conry et al. fails to teach or suggest all the elements of the claimed invention, as amended.

Moreover, the teaching of Conry et al. actually teaches away from a composition comprising a first vector encoding a cell surface receptor and a second vector encoding 4-1BB-ligand in combination with B7-1 or B7-2, as claimed. As described in Conry et al., "delivery of cDNAs encoding B7-1 and CEA within the same plasmid appears to augment the antitumor effects of low-dose CEA-polynucleotide immunization. In contrast, delivery of B7-1 cDNA on a separate plasmid has no detectable effect on tumor immunoprotection." Page 68, 2nd Col. (emphasis added). As further stated in Conry et al., "[o]ur studies using i.m.

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polynucleotide immunization demonstrated that co-delivery of B7-1 cDNA within a dual expression plasmid encoding CEA produced anti-CEA antibody responses and anti-tumor effects which were superior to those generated by plasmid DNA encoding CEA alone. However, co-delivery of B7-1 cDNA within a separate plasmid failed to enhance the immune response to plasmid DNA encoding CEA." Page 71, 1st Col. (emphasis added).

As mentioned above, Conry et al. does not teach the use of the 4-1BB-ligand. Moreover, the teaching of Conry et al. would lead one of skill in the art away from the use of a more that one vector to deliver an antigen and B7-1 to generate an antibody response, as claimed in the present invention. Therefore, it is demonstrated that the cited reference fails to disclose or suggest all the elements of the invention of Claim 7, from which Claims 9-20 depend. Removal of this ground of rejection is respectfully requested.

New Claims

Claims 9-20 have been added which depend from Claim 7. No new matter has been added. Support for the new claims is found throughout the specification as filed. Support for Claims 9, 10, 14, and 15 is found for example, at page 7, line 25, to page 8, line 14; and page 17, line 4, to page 18, line 10. Support for Claims 11 to 13 is found, for example, at page 8, line 15, to page 10, line 3. Support for Claims 16 and 17 is found, for example, at page 36, lines 1-5, and page 37, lines 18-23. Support for Claims 18 and 19 is found, for example, at page 48, lines 4-5; and page 49, line 29, to page 50, line 2. Support for Claim 20 is found, for example, at page 18, lines 12-15.

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CONCLUSION

In view of the foregoing, applicants submit that all of the pending claims are in condition for allowance and notification to this effect is respectfully requested.

Respectfully submitted,

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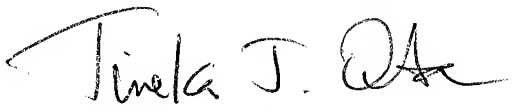

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Attachment 1

Plasmid-based vaccines encoding rat *neu* and immune stimulatory molecules can elicit rat *neu*-specific immunity

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Abstract

DNA vaccines are ideally suited for immunizing against tumor antigens because constructs can be formulated that not only encode the tumor antigen but also encode molecules chosen to improve the ability to elicit an antitumor response. Ligands expressed on antigen-presenting cells associated with stimulating a robust T-cell response are excellent candidates for inclusion in a DNA vaccine. Mice transgenic for the HER-2/*neu* homologue, rat *neu*, were immunized with full-length rat *neu* cDNA given alone or in combination with plasmids encoding costimulatory molecules CD80 or CD86 and the ligand for CD137 (CD137L). Intradermal injection of the plasmid constructs resulted in both plasmid transcript and antigen protein expression being detected in lymph nodes draining the injection site. Immunization with plasmids encoding the *neu* antigen along with plasmids encoding CD137L and either CD80 or CD86 resulted in the generation of *neu*-specific antibodies that induced phopshorylation of the *neu* tyrosine kinase and inhibited the growth of cultured tumor cells overexpressing *neu*. Survival of animals was significantly prolonged after immunization with vaccines encoding *neu* together with the costimulatory molecules. Although tumors eventually occurred in the vaccinated animals, they were markedly infiltrated with CD4+ T cells. DNA vaccines encoding *neu*, when given in combination with both CD137L and either CD80 or CD86, can induce cellular and humoral immunity and result in an antitumor effect. (Mol Cancer Ther. 2003;2:995–1002)

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Note: M.L.D. and N.S. contributed equally to this manuscript.

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Introduction

The development of tumor antigen vaccines in the form of bacterial plasmid DNA encoding an immunogenic protein has several potential advantages in clinical application as compared with more standard forms of immunization (1). First, DNA vaccines are not MHC restricted, in contrast to peptide vaccines. Secondly, DNA is easy to produce and transport and it is stable in long-term storage, in contrast to protein or viral vector-based vaccines. Finally, plasmid-based strategies may allow the delivery of multiple antigens simultaneously. Vaccinating against several tumor-related proteins would be beneficial as human cancers express multiple immunogenic proteins (2). Plasmid-based DNA vaccines, however, have been only minimally effective when translated to human clinical use. There are several reasons for the lack of success such as the low local transduction of DNA into antigen-presenting cells (APC) as most vaccines are delivered to muscle (3). Furthermore, monocytes and macrophages are more commonly transduced than dendritic cells (DC). In addition, APC transfected *in vivo* by DNA, generally in muscle, do not home effectively to draining lymph nodes; thus, systemic immunity is not efficiently stimulated (4). Intradermal (i.d.) immunization may be a more effective route of vaccination for plasmid DNA. Many APC are present in the epidermis and dermis including Langerhans cells and skin DC. *In vivo* transfection of skin cells with DNA encoding antigen as well as immune stimulatory molecules may allow the generation of more potent APC (*i.e.*, transform Langerhans cells or even dermal fibroblasts, monocytes, and macrophages into highly effective APC).

We have evaluated the efficacy of DNA immunization in the *neu* transgenic mouse. In this animal, rat *neu* is a homologue of human HER-2/*neu*, a well-defined tumor antigen in many solid tumors. HER-2/*neu* is a member of the epidermal growth factor receptor family and is presumed to function as a growth factor receptor (5). HER-2/*neu*, a transmembrane protein, consists of a cysteine-rich extracellular domain, which functions in ligand binding, and an intracellular cytoplasmic domain, which has kinase activity involved in growth regulation (6). In normal cells, it is present as a single copy (7). In contrast, the HER-2/*neu* gene is amplified in 20–40% of breast carcinomas and ~20–30% of ovarian carcinomas, and amplification occurs in other tumors, including carcinomas of uterus, stomach, and lung (8). The HER-2/*neu* protein is overexpressed in these tumors, and overexpression has been associated with a poor prognosis in carcinomas of the breast and ovary (9). In the mouse model described here, the *neu* transgene is expressed in the mammary gland and females spontaneously develop breast carcinomas (10).

From an immunologic standpoint, *neu* transgenic mice are tolerant to *neu*. Recent studies in the *neu* transgenic mouse suggest that both humoral and cellular *neu*-specific immunities are needed for tumor eradication (11).

We combined cDNA encoding full-length rat *neu* with cDNA encoding ligands of the costimulatory molecules CD28 and CD137. CD137 is expressed on both CD8+ and CD4+ T cells and recent studies demonstrate the costimulatory role of CD137 for CD4+ T cells (12–14). The CD137 costimulatory pathway is synergistic and yet independent of that mediated via CD28. In addition, CD137 engagement, by either antibody (15) or its ligand (16), can cause the rejection of established tumors, including those of low antigenicity (17). Intradermal vaccination with constructs encoding rat *neu* resulted in antigenic protein expression in the draining lymph node. The addition of costimulatory molecules to the vaccine induced the generation of both *neu*-specific antibodies and T cells, which resulted in prolonged survival in mice challenged with syngeneic *neu*-overexpressing tumors.

Materials and Methods

Construction of Plasmid-Based Vaccines

A plasmid encoding the *neu* antigen was constructed using pLNCX containing a cytomegalovirus (CMV) promoter (Fig. 1, A and B). Rat *neu* sequence was obtained by excising rat *neu* from pSV2 (5) with *Hin*dIII and *Afl*III. pLNCX-rat *neu* (RN) was constructed by cloning rat *neu* into *Hpa*I-cut pLNCX (Fig. 1B). Vaccination with the rat *neu* plasmid alone was used to establish the immunization route. Constructs encoding costimulatory molecules were generated using the multigenic cloning vector, pMG (InvivoGen, San Diego, CA), which contains two cloning sites, MCS1 and MCS2 (Fig. 1C), allowing a single plasmid to encode two costimulatory molecules (Fig. 1, D and E).

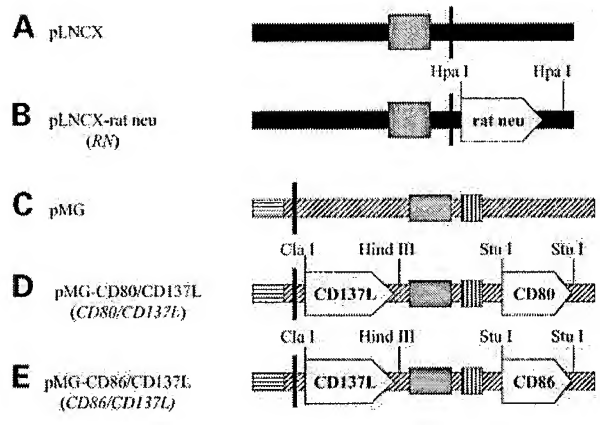

Figure 1. Plasmid construction. **A**, pLNCX expression cassette with MCS (*black bar*) and CMV promoter (*gray box*). **B**, pLNCX coding for full-length rat *neu* (*white arrow*). **C**, pMG expression cassette with MCS (*black bar*), hCMV-intron A promoter (*horizontal stripes*), encephalomyocarditis virus-internal ribosome entry site (*gray box*), and hEF-1-HTLV promoter (*vertical stripes*). **D** and **E**, pMG encoding CD80/CD137L and CD86/CD137L (*white arrows*).

The first transcriptional unit is under the control of an EF-1a/HTLV hybrid promoter, and the second unit contains a CMV promoter with intron A (18) and a modified internal ribosome entry site from encephalomyocarditis virus (19). Murine CD80 (20) was obtained by reverse transcription-PCR amplification of 5-day murine *Con*A blast RNA with the primers 5′ mCD80: TAAGCTTATGGCTTGCAATTGTCAGTTG and 3′ mCD80: GTATCGATCTAAAGGAAGACGG TCTGTTC. Murine CD86 (alternative variant B7.2I; 21) was obtained by reverse transcription-PCR amplification of 5-day murine *Con*A blast RNA with the primers 5′ mCD86: CGAAGCTTGTTCCAGAACTTACGGAAG and 3′ mCD86: CGATCGATCTTTCC TCAGGCTCTCAC. Murine CD137L was obtained by excision from pLXSHD (a gift from Dr. L. Chen, Mayo Clinic, Rochester, MN; 17) by *Sfi*I and *Eco*RI. Constructions of pMG CD80/CD137L (CD80/CD137L; Fig. 1D) and pMG CD86/CD137L (CD86/CD137L; Fig. 1E) required two cloning steps. First, linearization of pMG in MCS2 with *Pst*I-*Bam*HI and ligation of CD137L and then linearization of pMG CD137L in MCS1 with *Stu*I and ligation of Klenow-blunted CD80 or CD86 cDNA.

Immunization of *neu* Transgenic Mice with Plasmid-Based Vaccines

Specific pathogen-free breeder FVB/N-TgN (mouse mammary tumor virus-*neu*) 220 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at the University of Washington under specific pathogen-free conditions. Female mice 8–16 weeks old were used in the experiments described. Plasmids for DNA vaccination were prepared with Qiagen Plasmid Maxi Kit (Qiagen, Inc., Valencia, CA). The DNA was dissolved in water, stored at 4°C, and used within a month. In initial studies to define the dose and route of vaccine administration, mice were immunized s.c. in the flank and i.p., im., or i.d. in the flank or ear with varying doses of RN. Lymph node chains draining those injection sites were defined by sham injections with blue dye and the identification of the sentinel node (data not shown). In subsequent experiments where lymph node analyses were performed, those previously identified nodal chains were the focus of the evaluation. Groin nodes on the same side as the vaccination were harvested for flank immunizations, and cervical lymph nodes on the same side as the vaccination were harvested when injections were given in the ear. For vaccination experiments, groups of six mice were vaccinated once a week for 3 weeks with 30 μg of plasmid-based vaccine (30 μg of RN alone or 30 μg each of RN and CD80/CD137L or CD86/CD137L) diluted in 1× PBS (Dulbecco's PBS, Life Technologies, Inc., Grand Island, NY) and adjusted to a final volume of 50 μl. Mice were injected i.d. in either the flank or the ear. One week after the last vaccination, mice were transplanted sc. with 5×10^5 tumor cells from freshly removed spontaneous mammary carcinomas in *neu* transgenic mice. Tumor growth was assessed every 2 days via caliper measurement and the tumor surface was recorded (mm^2). Median survival times were compared with a two-tailed, paired *t* test using GraphPad

Instat (Version 3.05). Survival times were considered significantly different at $P < 0.05$. In addition, a mouse mammary carcinoma cell line (MMC) established from a spontaneous tumor and expressing high levels of rat *neu* was propagated *in vitro* and used for experiments described below.

Evaluation of Vaccine-Specific Transcription and Translation in Lymph Nodes Draining the Injection Site

The detection of pLNCX transcripts in lymph nodes by reverse transcription-PCR was calibrated *in vitro* using PE501, an ecotropic packaging cell line (22), retrotransfected with pLNCX (23). Transcripts were amplified from PE501 pLNCX cDNA with the primers P1: CCATCCACGCTGTTTTGACC and P2: CGTTACTTCCGCTAGCTTGCC using standard thermal cycling conditions. *In vivo*, DNA vaccine transcripts were detected by reverse transcription-PCR amplification of lymph nodes from immunized mice. After 48 h, the draining lymph nodes were harvested and immediately stored in RNA Later Solution (Ambion, Inc., Austin, TX) or directly processed. RNA was extracted with Trizol reagent (Life Technologies) following the manufacturer's protocol and 5 μg of the total RNA were reverse transcribed with Superscript II RNase H-Reverse Transcriptase (200 units/μl). The cDNA was amplified by PCR with the primers P1 and P2. Cytoplasmic expression of rat *neu* in lymph nodes before and 48 h after DNA vaccination was assessed by histologic techniques. Lymph nodes were harvested and embedded in paraffin. For staining, the sections were deparaffinized with HemoDe reagent (Fisher Scientific, Fairlawn, NJ), cleared with a graded alcohol series, and blocked with goat serum for 20 min. Sections were then incubated overnight at 4°C with 5 μg/ml of a rabbit anti-rat *neu* polyclonal antibody, c-*neu* Ab-1 (Oncogene Research Products, Cambridge, MA). After washing, the sections were incubated for 30 min in biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) and stained by 3,3'diaminobenzidine peroxidase substrate according to the manufacturer's instructions.

Quantitation and Functional Evaluation of Rat *neu*-Specific Antibodies

Rat *neu*-specific antibodies in serum were detected by modifications to an ELISA as previously described (24). Antibodies were functionally evaluated by phosphorylation assays and by their ability to inhibit tumor cell growth in soft agar. In the phosphorylation assays, MMC cells were seeded in 60 mm Petri dishes and grown to 70% confluence in culture medium. Sixteen hours before the assay, the cells were starved in RPMI supplemented with 0.2% FCS. After being washed once, the cells were incubated on ice for 5 min with 1:10 diluted sera from immunized mice and then immediately lysed with 10-mM Tris (pH 7.5), 150-mM NaCl, 1-mM EDTA, 1% Triton, Protease Inhibitor Cocktail Tablets (Complete, Mini, Roche Molecular Biochemicals, Indianapolis, IN) supplemented with 1 mM of sodium orthovanadate (Sigma). Nuclei were removed by centrifugation, 12,000 × *g* at 4°C, and the supernatants were immunoprecipitated with 10 μg/ml of c-*neu* Ab-4 (Oncogene Research Products) overnight at 4°C. Immunoprecipitated proteins were eluted following 3 h of incubation with Protein A-Sepharose and boiled in 2× SDS sample buffer (Novex, San Diego, CA) with 5% of 2-β-mercaptoethanol. Proteins were separated by electrophoresis on a 4–12% gradient gel (Novex), transferred to polyvinylidene fluoride membranes (Novex), and probed with horseradish peroxidase-conjugated antiphosphotyrosine antibody (4G10, Upstate Biotechnology, Inc., Lake Placid, NY) used in combination with the kit Western Breeze (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Signal was detected by enhanced chemiluminescence (Amersham, Piscataway, NJ) and quantified by using OptiQuant Version 03.00, May 12, 1998 program (Packard Instrument Co., Meriden, CT). After analysis for phosphorylated proteins, the blot was stripped and reprobed with c-*neu* Ab-4 (10 μg/ml) and horseradish peroxidase-conjugated anti-murine IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Inhibition of tumor growth by rat *neu*-specific antibodies was assessed using a modified protocol from Rapaport *et al.* (25). MMC were resuspended with a prewarmed solution of 0.6% ultralow gelling temperature agarose, Type IX-A (Sigma) in RPMI supplemented with 10% FCS and with 2-fold serial dilutions of sera from immunized mice. The cells were seeded into 60 mm Petri dishes coated with 1.2% low gelling agar suspension and incubated at 37°C for 15 days. Each sample was seeded in triplicate. Colonies comprising >30 cells were counted independently by two persons blinded to the experimental group.

Evaluation of CD4+ T-Cell Infiltration in Tumors

Sections of tumors from vaccinated animals were examined for infiltration of CD4+ T lymphocytes. Immunohistochemical staining was performed as described above, except that the sections were blocked with rabbit serum incubated with biotinylated anti-CD4 antibody (5 μg/ml; PharMingen, San Diego, CA). Furthermore, the second incubation step was conducted with 3,3'diaminobenzidine Vecta-Red peroxidase substrate (Vector Laboratories). CD4+ areas exhibit green fluorescence under dark-field microscopy.

Results

Intradermal Plasmid DNA Immunization Will Result in Both Plasmid Transcript and Antigenic Protein Expression in Draining Lymph Nodes

To initiate an immune response, antigen must be processed by APC, which traffic to a regional lymph node where antigen-specific T cells are stimulated. Fig. 2A demonstrates that DNA vaccination by the s.c. or i.p. route does not result in detectable plasmid in the lymph node draining the injection site. However, after the plasmid is given i.m. or i.d., transcript can be detected in the lymph nodes of immunized mice. Even at the lowest dose of plasmid (1 μg), pLNCX could still be identified in the draining lymph node of mice vaccinated via the i.d. route. To determine whether trafficking cells expressed rat *neu*

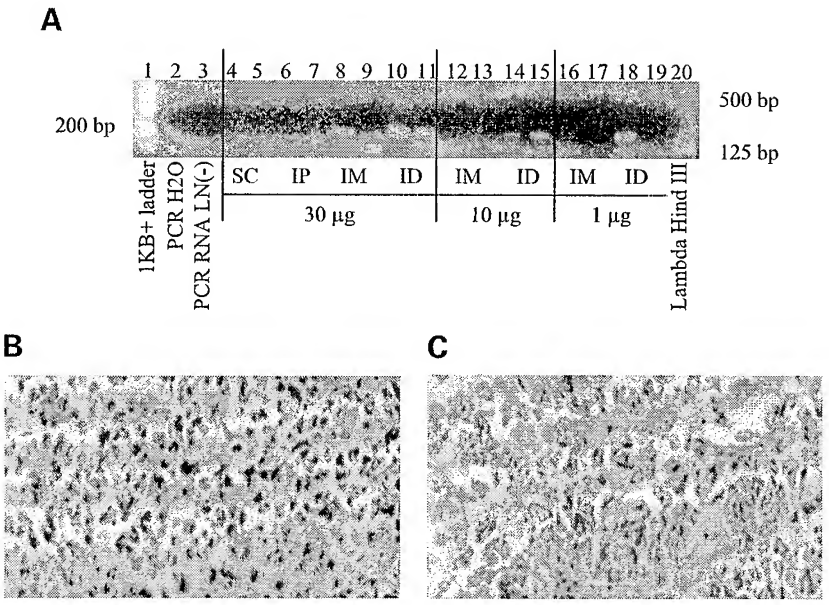

Figure 2. Intradermal plasmid DNA immunization will result in both plasmid transcript and antigenic protein expression in draining lymph nodes. **A,** PCR of cDNA from draining lymph nodes 48 h after injection. *Lanes 4–11,* individual animals immunized with 30-µg RN: s.c. vaccination (*4 and 5*), i.p. (*6 and 7*), i.m. (*8 and 9*), and i.d. (*10 and 11*). For subsequent doses, only the i.m. and i.d. routes were evaluated. *Lanes 12–15,* lymph nodes from mice that received 10-µg RN: i.m. (*12 and 13*) and i.d. (*14 and 15*). *Lanes 16–20,* 1-µg vaccinations: i.m. (*16 and 17*) and i.d. (*19 and 20*). *Lanes 1 and 20,* size markers. Negative controls were identical PCR reactions with H_2O (*lane 2*) or cDNA from lymph nodes of naïve mice (*lane 3*). Representative of three independent experiments (2 mice/condition). **B** and **C,** sections from lymph nodes stained for rat *neu* protein expression at 48 h after injection. Lymph nodes were derived from animals vaccinated with pLNCX (**B**) or RN (**C**). Representative of three independent experiments.

protein, we performed immunohistochemical staining of lymph nodes derived from mice immunized i.d. with either 30 µg of pLNCX or RN. Fig. 2B demonstrates that mice receiving plasmid alone did not show evidence of protein expression in the draining lymph node. However, mice receiving plasmid that encoded rat *neu* had significant expression of the rat *neu* protein in the cytoplasm of cells in the lymph node (Fig. 2C).

Addition of Costimulatory Molecules to Plasmid DNA Immunization Elicits a Functional *neu*-Specific Antibody Response after Vaccination

Antibodies to rat *neu* were assessed by ELISA. Fig. 3 demonstrates that rat *neu*-specific antibodies could not be detected in animals immunized with the pLNCX or pMG vectors alone. A significant antibody response was defined as the mean ± 2 SD of the *neu*-specific antibody response in six control (PBS-immunized) animals (0.03 ± 0.02). Low-titer (≤1:200) rat *neu*-specific antibodies could be detected in mice immunized with RN or RN + pMG, although the addition of pMG to RN did not enhance the response (Fig. 3). In contrast, significantly higher-titer (>1:800) *neu*-specific antibodies could be detected in mice vaccinated with RN + costimulatory molecules as compared with mice immunized with RN or RN + pMG, RN + CD80/CD137L ($P < 0.001$ at all dilutions), or RN + CD86/CD137L ($P < 0.001$ at all dilutions). Activated *neu* induces a phosphorylation cascade in cytoplasmic kinases (26, 27). To determine whether the rat *neu*-specific antibodies elicited with active immunization could affect signaling via the tyrosine kinase, MMC cells, which overexpress rat *neu*, were incubated with sera from naïve mice and from mice immunized with RN + either CD80/CD137L or CD86/CD137L. As shown in Fig. 4A, serum from mice immunized with rat *neu* with costimulatory molecules induced tyrosine phosphorylation of the rat *neu* protein, while sera from naïve mice or from mice immunized with RN (and RN + pMG, data not shown) did not have an effect on phosphorylation. The increase in phosphorylation was not related to different concentrations of the *neu* receptor in the preparations (Fig. 4A).

We then assessed whether sera from immunized mice, which affected *neu* receptor tyrosine phosphorylation, could also impact anchorage-independent tumor cell growth. MMC tumor cells were exposed to sera and colony formation was assessed with soft agar assays. Fig. 4B shows that sera from animals immunized with RN ($P = 0.01$ at 1:40 dilution), RN + CD80/CD137L ($P = 0.003$), or RN + CD86/CD137L ($P = 0.001$) could significantly inhibit colony formation when compared with sera from naïve mice. While immunization with RN was effective at

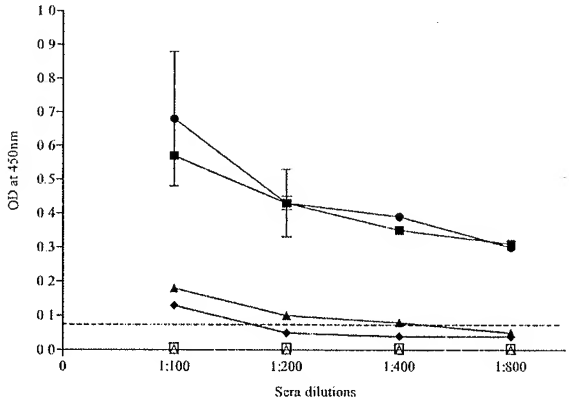

Figure 3. Rat *neu*-specific antibody immunity can be generated after immunization with plasmid-based vaccines encoding costimulatory molecules. Mice were immunized thrice with vaccine constructs and sera were obtained 45 days after the last vaccine. Antibody titers for mice immunized with PBS (△), pLNCX (□), or pMG (○). Antigen-containing vaccines included RN (♦), RN + pMG (▲), RN + CD80/CD137L (■), or RN + CD86/CD137L (●). *Dotted line,* mean ± 2 SD of the nonimmunized controls. *Lines,* mean; *bars,* SD (6 mice/group).

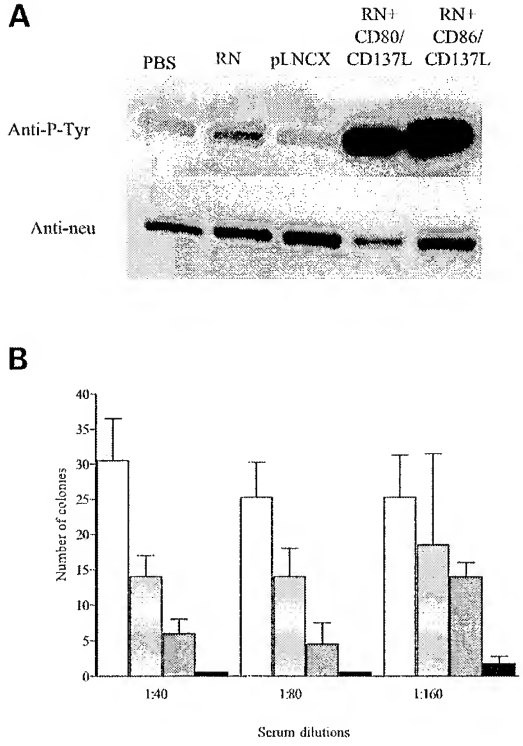

Figure 4. Rat *neu*-specific antibodies can induce rat *neu* tyrosine phosphorylation and inhibit the growth of *neu*-overexpressing tumor cells in vitro. **A,** (*top*) phosphorylation of *neu* expressed in MMC after treatment with sera derived from animals immunized with PBS, RN, pLNCX, RN + CD80/CD137L, and RN + CD86/CD137L; (*bottom*) *neu* protein from treated cells is present at similar levels. Representative of three independent experiments. **B,** MMC cells incubated with varying dilutions of experimental sera: naive mice (*white*), RN (*light gray*), RN + CD80/CD137L (*dark gray*), and RN + CD86/CD137L (*black*). *Columns*, mean of triplicate cultures; *bars*, SD. Representative of three individual experiments.

inhibiting colony formation, the addition of either CD80/CD137L ($P = 0.02$ *versus* RN) or CD86/CD137L ($P = 0.002$ *versus* RN) significantly improved the inhibitory effects of RN immunization. Furthermore, CD86/CD137L was superior to CD80/CD137L ($P = 0.01$).

Plasmid DNA Immunization Targeting Rat *neu* Delays Tumor Development *in Vivo* and the Effect Is Augmented by the Addition of Costimulatory Molecules to the Vaccine

Mice were immunized with PBS alone, RN, RN + CD80/CD137L, or RN + CD86/CD137L, and a month after the final vaccination, 5×10^5 syngeneic tumor cells were implanted. Forty-five days after implantation, tumors could be detected in the PBS control group and measurements were taken every 2 days thereafter (Fig. 5). Although all the immunized groups developed tumors, animals vaccinated with RN + CD86/CD137L had a significantly prolonged survival over both PBS-immunized mice ($P < 0.0001$) and mice immunized with RN ($P = 0.0007$). The difference in survival between animals immunized with RN + CD80/CD137L and RN + CD86/CD137L was not significant ($P = 0.12$). Tumors from immunized mice contained marked CD4+ T-cell infiltrates. Fig. 6 depicts immunochemically stained sections of tumors from mice given PBS (Fig. 6A) or immunized against RN (Fig. 6B), RN + CD80/CD137L (Fig. 6C), or RN + CD86/CD137L (Fig. 6D). In animals immunized with RN + CD86/CD137L, tumor destruction and necrosis is evident (Fig. 6D).

Discussion

Potent methods of immunization are particularly important when contemplating vaccinating against cancer. Most of the recently identified tumor antigens are self-proteins and tolerance to self is one of the major mechanisms of tumor immune escape (28–30). Cancer vaccine strategies must be developed that are capable of augmenting or generating immune responses against self-tumor antigens (*i.e.*, circumvent tolerance). Considerable effort has been focused on using potent APC such as DC to process and present tumor antigens to the immune system (31–33). *Ex vivo* generation of DC, however, is laborious and may not be practical for wide-scale vaccine applications (34, 35). Our strategy for vaccinating against a self-tumor antigen, rat *neu*, in the *neu* transgenic mouse focuses on *in vivo* modification of skin cells with plasmid DNA constructs encoding costimulatory molecules chosen to enhance immune recognition. Data presented here demonstrate that (1) i.d. injection of plasmid DNA encoding *neu* will result in antigen expression in the local draining lymph node, indicating cells that traffic from skin to lymph node have been transfected *in vivo*; (2) functional *neu*-specific antibody immunity can be generated when *neu* encoding constructs are used in active immunization in conjunction with constructs encoding costimulatory molecules CD137L and CD80 or CD86; and (3) use of CD137L and CD80 or CD86 as costimulatory molecules in plasmid DNA vaccines will result in marked CD4+ T-cell infiltrates in tumors and significantly prolong survival in vaccinated mice.

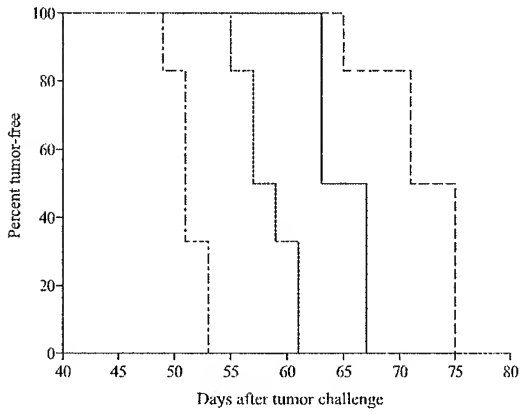

Figure 5. Plasmid DNA immunization targeting rat *neu* can delay tumor growth and the effect is augmented with the addition of costimulatory molecules in the vaccine. Groups of six mice were immunized with DNA vaccines. pLNCX (-●-), RN (—), RN + CD80/CD137L (–), and RN + CD86/CD137L (– – –). Percent survival of mice is plotted as a function of the time after tumor implantation.

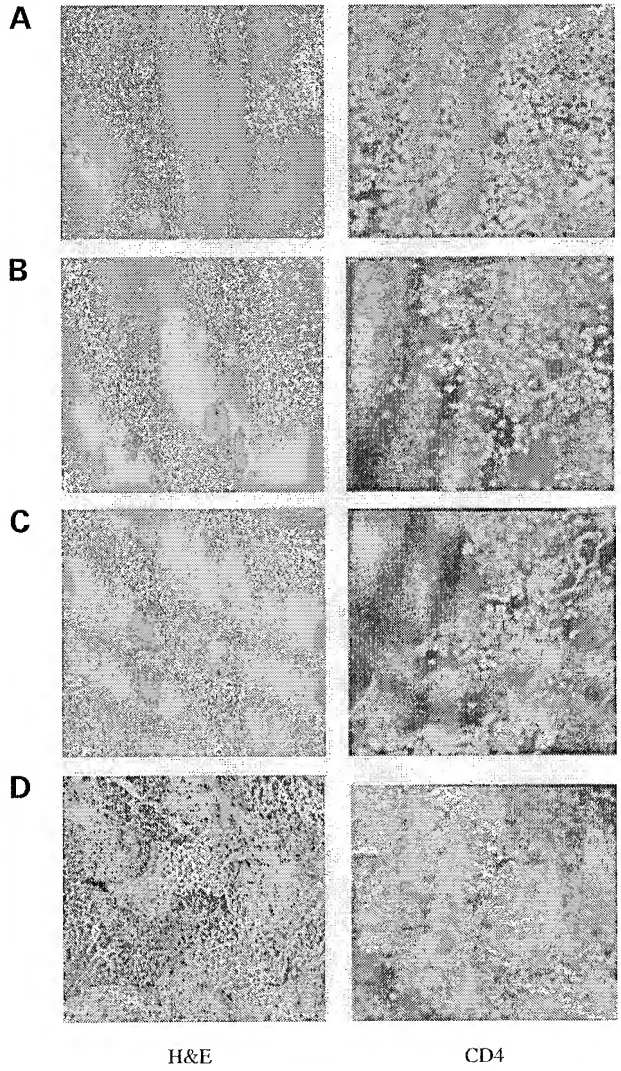

Figure 6. Tumors derived from mice immunized with rat *neu*-specific DNA vaccines contain CD4+ T cells. Tumors derived from immunized mice were stained with H&E (*first column*) or anti-CD40 monoclonal antibody (*second column*). Tumors from mice immunized with pLNCX (**A**), RN (**B**), RN + CD80/CD137L (**C**), and RN + CD86/CD137L (**D**). Data from an individual tumor are representative of 6 animals/experimental group.

For DNA vaccination to be successful, antigen encoded must be effectively taken up and processed by APC and presented to T cells in the draining lymph node. Therefore, activated APC must internalize the antigen at the site of injection and have the ability to migrate to the local lymph node. In addition, immunostimulatory molecules should be up-regulated on antigen-bearing APC to enhance presentation of antigen to T cells, thus stimulate a systemic immune response. In our model, i.d. immunization was the most effective route of vaccination for demonstrating antigen expression in the lymph node draining the injection site. Even at the lowest doses of plasmid DNA delivered, vector could still be detected in the lymph node. Vector transcript was only found at the injection site and in the draining lymph node and not in brain, heart, lungs, spleen, liver, digestive tract, or kidney (data not shown). Therefore, detection of plasmid and *neu* antigen expression in the lymph node is most likely due to locally transfected cells in the skin migrating to the node rather than a nonspecific generalized tissue distribution of the plasmid. APC in the skin capable of trafficking to lymph nodes include Langerhans cells or other nonprofessional APC such as macrophages. The use of costimulatory molecules in the plasmid constructs may ensure that even nonprofessional APC express molecules capable of triggering T-cell activation.

We chose to evaluate CD137L, CD80, and CD86 as costimulatory molecules to include in plasmid constructs to be delivered along with the *neu* antigen. CD137 is a member of the tumor necrosis family receptors and is expressed on activated T cells as well as on NK cells and monocytes for which it serves as a survival factor (36). CD137L is expressed on activated APC and has been shown to stimulate both CD4+ and CD8+ T cells (37). Although the CD137 axis is associated with the generation of a CD8+ T-cell response, it has been shown that CD137L can enhance the function of either CD4+ or CD8+ T cells with similar efficacy (12, 38). Furthermore, CD137L interactions with activated T cells will result in continued numerical and functional expansion of these immune effector populations *in vitro* (37). The CD137 signaling axis may be particularly important in the generation of an antitumor response. Recent studies have demonstrated in a poorly immunogenic murine tumor model that triggering signaling via CD137 in the presence of specific tumor antigen will result in tumor regression (39, 40).

Important for stimulating a naïve T-cell response, T-cell costimulatory receptors CD28 and CTLA-4 bind CD80 and CD86 ligands on APC (41). In addition, the CD28 pathway is critical in the maturation of the B-cell response (42). Several strategies have been developed to use these ligands in combination to enhance antigen-specific immune responses *in vivo* or *in vitro*. Investigators have transfected lymphoma cells with CD80, CD86, or CD137L and evaluated whether the intact tumor cell could act as a cancer vaccine (43). Although tumor cells transfected with CD80 or CD86 conferred partial protection, those including the CD137L resulted in long-lasting tumor protection. More recent studies have demonstrated that transfecting cells with the ligands for CD28 and CD137 results in an artificial APC that is capable of directly stimulating or recruiting cells that could stimulate the expansion of human T lymphocytes *in vitro* (44). Our strategy was to create such artificial APC *in vivo* in the skin by direct i.d. transection.

The *neu* transgenic mouse is a model for simulating human malignancy as mice are tolerant to *neu*. Plasmid-based vaccines encoding *neu* and CD137L and either CD80 or 86 were effective in generating both an antibody and a T-cell response specific for *neu*; therefore, tolerance was circumvented. The tumor antigen in our system, *neu*, is a growth factor receptor and antibodies directed against *neu* may mediate a therapeutic effect. Indeed, *neu*-specific antibodies elicited after active immunization induced tyrosine phosphorylation. Many *neu* ligand mimicking

antibodies will stimulate phosphorylation of the *neu* tyrosine kinase and their associated antitumor effect correlates with accelerated endocytic degradation (45, 46). The antibodies elicited after active immunization could suppress the growth of *neu*-overepxressing tumor cells *in vitro*. Although survival was prolonged in mice vaccinated with DNA encoding *neu* and CD137L and CD80 or CD86, tumors eventually developed. In studies of active immunization in human melanoma, it has been noted that there can be a dissociation between local and systemic immune responses against a specific tumor antigen (47). For this reason, we assessed the ability of T cells to home to antigen-bearing tumor as a functional measure of immunity. The tumors that did develop in the immunized animals were infiltrated predominantly with CD4+ T cells and tumors derived from the mice vaccinated with constructs encoding CD137L and CD86 demonstrated areas of necrosis. Although one could expect that CD8+ T cells could also be generated by endogenous processing via class 1 pathway by internally expressed protein, the infiltrating T cells were primarily CD4+. An immune response was generated but it was not fully protective. Investigations by others have demonstrated that the immunostimulatory properties of CD137L can be enhanced by the addition of cytokines such as interleukin-12 (48). The experiments performed here used the plasmid DNA constructs without any additional adjuvant. Potentially, the vaccine could be made more effective by coadministration with soluble cytokines such as interleukin-12 or granulocyte macrophage colony-stimulating factor (49).

Multiple tumor antigens have been defined, especially for common malignancies such as breast cancer (2). Recent studies have shown that patients with breast cancer can be immunized against proteins expressed by their tumors including HER-2/*neu* (50, 51). Vaccine technologies must be developed that will allow an immune response to be generated to multiple antigens expressed in a patient's tumor. Plasmid-based vaccine strategies offer such a technology that is easily and clinically applicable in a broad population. Costimulatory molecules functioning to transform local APC in the skin to stimulate immunity to weak antigens, such as a self-tumor antigen, have the potential to make plasmid-based tumor antigen vaccines therapeutically effective.

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